IDENTIFICATION OF MYCOBACTERIUM TUBERCULOSIS USING CRISPR-CAS12A

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ABSTRACT

Tuberculosis (TB) is a global health concern and a communicable disease. It was the leading cause of death from a single infectious agent before the COVID-19 pandemic, surpassing HIV/AIDS. Mycobacterium tuberculosis (MTB) is the causative agent of TB, which spreads through airborne transmission, primarily via coughing. However, the conventional method for identifying MTB is time-consuming and limited to large hospitals, resulting in delays in targeted treatment and challenges in reaching affected communities. Therefore, there is an urgent need for a rapid and easy-to-use approach to detect MTB, especially in resource-limited areas. In this study, we investigated the use of CRISPR-Cas12a-based detection as an alternative and promising method for TB screening. We developed a specific detection method for MTB using the CRISPR system combined with recombinase polymerase amplification (RPA) to enhance signal amplification. The results demonstrated successful amplification and detection of the rpoB target gene of MTB using the designed primers and crRNA. Importantly, these assays exhibited no cross-reactivity with other Mycobacterium species, and this technique had a limit of detection (LOD) as low as 10^3 copies/µL. In conclusion, the RPA combined with CRISPR-Cas12a assay proved to be an effective screening method for MTB, providing results in less than 1 hour from extracted DNA. This assay holds significant potential as a portable, rapid, and cost-effective MTB detection tool, especially in low-resource settings.
Keywords: Tuberculosis, Mycobacterium tuberculosis, CRISPR-cas12a, RPA

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INTRODUCTION

Tuberculosis (TB) is a contagious infectious disease caused by the bacteria Mycobacterium tuberculosis (MTB). MTB can disseminate to different parts of the body through direct spread from the lungs, where the primary infection usually occurs. It can also spread through the lymphatic system, or bloodstream, resulting in various pulmonary and extrapulmonary manifestations of TB. In pulmonary TB, the infection primarily affects the lungs and is characterized by symptoms such as persistent cough, coughing up blood, chest pain, and difficulty breathing. However, if the bacteria enter the bloodstream or lymphatic system, it can reach other organs or tissues and cause extrapulmonary TB. Extrapulmonary TB can affect areas such as the kidneys, bones and joints, spine, brain, lymph nodes, and other organs. The symptoms and manifestations of extrapulmonary TB vary depending on the site of infection (Bloom et al., 2017). TB is a global disease, found in every country in the world. It is one of the leading infectious causes of death worldwide. In 2021, an estimated 1.6 million people died of TB Including 187,000 people with HIV, making it the 13th leading cause of death worldwide. Notably, TB remains the second leading infectious killer, surpassing HIV/AIDS, with only COVID-19 surpassing it in terms of mortality (Bagcchi, 2023). According to the World Health Organization's report, it has been found that the incidence of TB is increasing both in developed and developing countries. It predominantly affects populations residing in the regions of South-East Asia, Africa, Western Pacific, with a smaller percentage in the Eastern Mediterranean, the Americas, and Europe. The primary contributing factors to the persistent and severe tuberculosis epidemic are primarily attributed to the failure in controlling and preventing the disease, which may arise from inadequate disease surveillance, diagnosis, and inappropriate treatment (Chakaya et al., 2021). Therefore, an effective diagnostic approach that includes appropriate laboratory testing methods, imaging x-rays studies, and the use of antimicrobial drugs to target MTB, is essential to plan treatment for patients with TB and stop the spread of TB (Pai et al., 2016).

In general, the identification of MTB is based on conventional methods, including examination under a microscope with acid-fast bacilli (AFB) staining using Ziehl-Neelsen (Z-N) and determination of bacterial morphology. Z-N staining is a quick, easy, and inexpensive method but has low specificity and cannot distinguish between the MTB and other non-tuberculous mycobacteria (NTM). Additionally, the gold standard for laboratory diagnosis is isolating Mycobacterium from clinical samples, but culture using Lowenstein-Jensen (LJ) medium is a slow and difficult process that typically takes 4-8 weeks for confirmation. Biochemical tests, such as niacin, catalase, nitrate reduction, and urease tests, are also often used to identify species, but they are time-consuming and cannot differentiate between closely related species. These testing methods are complex and labor-intensive. For these reasons, methods have been developed to detect and identify MTB that is rapid, simple, sensitive, and specific (Campelo et al., 2021). In recent decades, molecular approaches have been developed to diagnose TB. Nucleic acid amplification-based tests such as PCR, GeneXpert MTB/RIF assay, and line probe assay have greatly improved the sensitivity, specificity, and accuracy of mycobacterial diagnosis. However, despite their widespread use, these techniques require expensive laboratory equipment and the chemicals used are costly and must also be imported from abroad. Furthermore, the amplification and detection steps of the assay involve multiple steps which may introduce the potential for error and sample contamination (Nurwidya et al., 2018).

Recently, a clustered regularly interspaced short palindromic repeat (CRISPR-Cas) system has been successfully developed and demonstrated. This system consists of a Cas effector, guide RNA (gRNA), nucleic acid amplification mixture, CRISPR-Cas buffer, and reporter molecules (single-strand nucleic acid). For example, CRISPR-Cas12a-based HOLMES (One-Hour-Low Cost Multipurpose Highly Efficient System) and CRISPR-Cas13a-based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) assays have been developed for

the diagnosis of SARS- CoV-2, HPV (human papillomavirus), ZIKV (Zika virus), and DENV (Dengue virus) with attomolar sensitivity. In these target gene assays, crRNAs must be designed to match the target nucleotide sequence, which recruits Cas proteins to the target site and triggers nonspecific nucleotide cleavage. The activated Cas proteins then cleave a nucleotide reporter containing fluorescence and quencher. The cleavage unleashes a fluorescence signal that enables detection of the target gene. The difference between the two systems is that Cas12 recognizes double-strand DNA (dsDNA) as an activator and cleaves single-strand DNA (ssDNA), while Cas13 recognizes ssRNA and cleaves ssRNA. However, CRISPR-Cas12a and CRISPR-Cas13 provide high sensitivity and specificity (Chen et al., 2018; Gootenberg et al., 2017). Therefore, the objective of this study is to develop a combined RPA and CRISPR-Cas12a system for the identification of MTB. The primary goals of this research are to enhance assay performance and enable the rapid detection of MTB, ultimately facilitating the timely diagnosis of TB.

LITERATURE REVIEWS

Mycobacterium tuberculosis

Mycobacterium tuberculosis (MTB) is a pathogenic bacterium in the family Mycobacteriaceae that causes tuberculosis (TB), a contagious bacterial infection that primarily affects the lungs. It is a member of the Mycobacterium tuberculosis complex (MTBC), which includes several closely related species that can cause TB in humans and animals (Bañuls et al., 2015). MTB is a rod-shaped bacterium that appears as slightly curved or straight rods under a microscope. It has a unique cell wall composed of a complex lipid-rich structure called mycolic acids, which makes it acid-fast staining property and resistant to chemicals and host immune responses. This bacterium is an aerobic and slow-growing bacterium, which means that it takes several weeks to form visible colonies on culture media. This slow growth rate poses challenges to diagnosis and treatment of TB (Babalola, 2015). Additionally, it possesses several virulence factors that contribute to its pathogenicity. These include the ability to inhibit the fusion of phagosomes with lysosomes, leading to intracellular survival within host cells such as macrophages, and the production of factors that modulate host immune responses, and cause disease (Forrellad et al., 2013; Rahlwes et al., 2023). MTB is transmitted through the air when infected individuals cough, sneeze, or speak, releasing infectious respiratory droplets. Common symptoms of TB include a persistent cough, fever, night sweats, weight loss, and fatigue. It has the ability to develop resistance to antibiotics used for TB treatment. This multidrug resistance (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) pose significant challenges to TB control and treatment (Chakaya et al., 2021).

The standard methods for identification of MTB

The standard methods for the identification of MTB, the causative agent of TB, involve a combination of laboratory techniques. Acid-fast staining is a widely used method that involves staining clinical samples, such as sputum or cerebrospinal fluid, with specific dyes such as the Ziehl-Neelsen or Kinyoun stain. Acid-fast bacilli, including MTB, retain the stain even after being washed with an acid-alcohol solution, allowing for their visualization under a microscope. This staining method provides a preliminary indication of the presence of mycobacteria. However, smear microscopy does not identify the mycobacterial species or provide information about the viability of the mycobacteria in the sample. Patients with tuberculosis co-infected with HIV may have a paucibacillary form of the disease with fewer acid-fast bacilli, making smear microscopy less reliable in these cases. As a result, smear microscopy may require more scrutiny or may be negative in the screening for fewer acid-fast bacilli (Huang et al., 2022).

Culture is considered the gold standard for confirming the presence of MTB. It involves inoculating a clinical specimen onto specialized media that support the growth of mycobacteria.

The most used solid medium is Lowenstein-Jensen (LJ) agar, which contains specific nutrients that promote the growth of MTB. Liquid media such as Middlebrook 7H9 broth are also utilized. Culturing MTB can take several weeks, as the bacterium has a slow growth rate. However, once colonies of MTB are obtained, they can be further subjected to various tests for identification and drug susceptibility (Caulfield & Wengenack, 2016).

Currently, The World Health Organization (WHO) recommends the use of molecular methods, specifically nucleic acid amplification tests (NAATs), for the identification of TB. NAATs are highly sensitive and specific tests that detect the presence of the MTB. One of the commonly used NAATs for TB diagnosis is the GeneXpert MTB/RIF assay, which utilizes real-time polymerase chain reaction (PCR) technology. This assay simultaneously detects the DNA of MTBC and also provides information about the presence of resistance to rifampicin, an important first-line anti-TB drug. Rifampicin resistance is an indicator of multidrug-resistant TB (MDR-TB). It can detect mutations in the rpoB gene that cause resistance to rifampicin. It has a sensitivity of 94.4% and a specificity of 98.3% (Sailo et al., 2022). The test takes approximately 2 hours to detect in the sputum samples, regardless of whether the AFB smear results are positive or negative. However, when choosing to use the Xpert MTB/RIF test, it is important to consider that the operation of the detector requires a constant power supply for at least 2 hours, that the detector can only be used at a temperature not exceeding 30°C, and the storage cartridges should be stored at a temperature between 2-8°C. Additionally, it is not suitable for monitoring a patient's response to treatment and the costs of maintenance and calibration are expensive (Gidado et al., 2018; Kohli et al., 2021).

CRISPR-Cas systems

The CRISPR-Cas systems composed of clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins play a role in the adaptive immunity of archaea and bacteria against foreign nucleic acids. In recent years, CRISPR/Cas technology has emerged as a powerful tool for editing genomes in all forms of life that have been adapted for various applications, including the diagnostics (Parsaeimehr et al., 2022). The CRISPR-Cas system utilizes guide RNA to direct specific Cas effector proteins (such as Cas12, and Cas13) to particular target sites. If the genetic material is present in the sample, the CRISPR-Cas enzymes will bind to it and become activated. Once activated, the CRISPR-Cas enzymes will induce a fluorescence signal or produce a detectable output, indicating the presence of the target (Freije & Sabeti, 2021). Recent advancements in CRISPR technology have expanded its applications to include nucleic acid detection using Cas12a. CRISPR-Cas12a is being used to detect a variety of viral strains, such as SARS-CoV-2, influenza viruses, HPV, pseudorabies virus, and Japanese encephalitis virus (Gao et al., 2022; Mayuramart et al., 2021). Some groups have utilized PCR for DNA amplification. However, this method necessitates multiple cycles of heating and cooling to denature and anneal DNA strands. Conversely, LAMP (Loop-Mediated Isothermal Amplification) is an isothermal amplification technique that operates at a constant temperature, typically around 60-65°C. Nonetheless, LAMP requires a minimum of four primers to effectively amplify a target DNA sequence (Oliveira et al., 2021). Additionally, recombinase polymerase amplification (RPA) is another preferred amplification technique because it can be performed isothermally between 37 and 42°C, close to the optimal temperature for the Cas12a cleavage assay (37°C) and requires only two primers. RPA has been used in various diagnoses and applied in clinical settings with limited resources. RPA has advantages such as being simple to use, low time-consuming, low-cost, and focused on point-of-care techniques (Mayuramart et al., 2021).

Currently, an assay has been utilized for the detection of specific Mycobacterium species as M. tuberculosis and M. abscessus using techniques that amplify the target DNA through PCR or LAMP, which require complex instrumentation and handling (Sam et al., 2021; Xiao et al., 2020). Although there have been studies employing a combination of RPA and CRISPR-Cas

for MTB detection, they have limitations in terms of visual readout as the Cas12a trans-cleavage fluorescence is detected by a fluorescence detector (Xu et al., 2020). Therefore, this study aims to develop a CRISPR-Cas12a protocol in combination with RPA for the rapid detection of MTB. This assay offers the advantages of easy operation, quick turnaround time, and suitability as a point-of-care clinical diagnostic test. It can be particularly valuable in resource-limited settings for TB, facilitating specific and timely treatment planning by healthcare professionals.

RESEARCH METHODOLOGY

Genomic DNA extraction from reference strains

Heat-killed MTB and other NTM species isolates were centrifuged at 10,000 g for 10 minutes to obtain pellets. The pellets were then used to extract genomic DNA using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, California, USA) following the manufacturer's instructions or the CTAB method. The extracted DNA was stored at -80°C until use.

Primers and CRISPR RNA

The primers and CRISPR (crRNA) were designed to target the highly specific rpoB gene for MTB. DNA sequences of known variable genes from NGS base BTSeq^{TM} barcode-tagged sequencing (U2BIo, Thailand) were aligned, and conserved regions were identified for amplification and detection. A primer pair for the amplification of target sequences were selected using agarose gel electrophoresis, according to the TwistAmp Assay Design Manual (TwistDx Ltd, Cambridge, MA). The oligonucleotides for the crRNA template for in vitro transcription was prepared via overlap extension PCR. Briefly, two short DNA oligos, one containing the T7 promoter and the other containing the spacer were designed. The T7 promoter was used as the starting point for the formation of crRNA strands by the enzyme T7 RNA polymerase, which was initiated by adding the nucleotide sequence of the T7 promoter (T7 LbCpf1 crRNA Top; 5' TAATACGACTCACTATAGtAATTTCTACTAAGTGTAGAT '3) into the scaffold. The oligonucleotides for the crRNA template primer were annealed to the T7 promoter primer and incubated under the following conditions; 95°C for 1 min, 95°C for 30 sec, 58°C for 15 sec, 72°C for 15 sec, repeat 35-40 cycles and 72°C for 1 min. The crRNA was then transcribed using Riboprobe® in vitro transcription systems (Promega, USA) according to the manufacturer's protocol. Transcribed crRNA was purified using the miRNA isolation kit (Geneaid, Taiwan) and quantified using the Qubit TM microRNA Assay Kit (Thermo Scientific).

RPA amplification of rpoB gene

RPA amplification of rpoB gene which contains a region of deletion. These was used TwistAmp® Basic kit (TwistDx, Cambridge, UK). A total volume of 10 µL of RPA containing 5.9 µL of rehydration buffer with RPA powder, 0.48 µL of 10 µM of each forward and reverse primer (Table 1), and 2.14 µL of distilled water. Then, the solution was mixed and added to freeze-dried reagent lyophilize. After that, the mixture was vortexed and added 0.5 µL of 280 mM magnesium acetate on the tube lid before adding 1 µL DNA template. Finally, the RPA reaction was incubated at 39°C for 40 min followed by heat inactivation at 75°C for 5 min in a heat box.

Limit of detection

The limit of detection was performed in triplicate by doing a 10-fold serial dilution of each standard DNA target ranging from 1 to 10^5 copies/µL as templates for RPA with CRISPR-Cas12a. The limit of detection was observed from reaction tubes containing the lowest concentration of the DNA template that yielded the fluorescent signal.

CRISPR-Cas12a detection

The reaction of CRISPR-Cas12a-based nucleic acid detection consisted of 30nM crRNA (Table 1), 33nM EnGen Lba Cas12a (Cpf1) (New England Biolabs, USA), 660nM fluorescent

reporter/quencher probe, 1x reaction buffer NEBuffer 2.0 (New England Biolabs, USA), and 1 µL of RPA product in a final volume of 15 µL. The Cas reaction was incubated at 39°C for 15 min and then the fluorescent signal was visualized with a BluPAD dual LED blue/white light transilluminator (BIO-HELIX, Taiwan). Negative controls or no template control (water, DNase, and RNase-free) were used in parallel each time. The interpretation of the CRISPR-Cas assay was recorded from three independent persons and then the concordant results obtained from a least two of these interpreters were used as the final outcome. The collected data was then analyzed.

Table 1 Oligonucleotides are used for RPA and crRNA production.

Primer's Name	**Sequences (5′→3′)**
rpoB1-F	GACATCCTGGTCGGCAAGGTCACCCCGAAGGG
rpoB2-F	CTCCGTACCCGGAGCGCCAAACCGGGTCTCCTTCGC
rpoB3-F	AATAACTCCGTACCCGGAGCGCC
rpoB4-F*	AATAACTCCGTACCCGGAGCSCC
rpoB1-R	AGCTTGTCACCGTCGGAGATCTTGCGTTTCTG
rpoB2-R*	GCCGCGTACGTCATGTCCTTGTCTTTGCACTCGTCG
TB-1	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGAT GACGATGTCAAGGCACCCGT
TB-2	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGAT GACGATGTCACGGCACCCGT
ssDNA-FQ	FAM-AGGACCCGTATTCCCA-BQH1

Note: Underline sequence represents spacer sequence. ssDNA-FQ was used in the CRISPR-cas12a detection trans-cleavage fluorescence assay. The rpoB-F and rpoB-R are the forward and reverse primers of RPA, respectively. The final sequences of RPA primers are marked (*). FQ, fluorophore quencher; TB M. tuberculosis crRNA; rpoB the beta subunit of RNA polymerase (target gene).

RESEARCH RESULTS

To identify MTB using RPA-CRIPSR Cas detection, the rpoB gene encoding the beta subunit of RNA polymerase is first amplified. Then, the RPA product is incubated with Cas12a, crRNA, and ssDNA fluorescence reporter. If the specific crRNA probe in the reaction mixture detects the target DNA, the Cas12a/gRNA/target DNA ternary complex will form and cleave the ssDNA reporter, generating fluorescence.

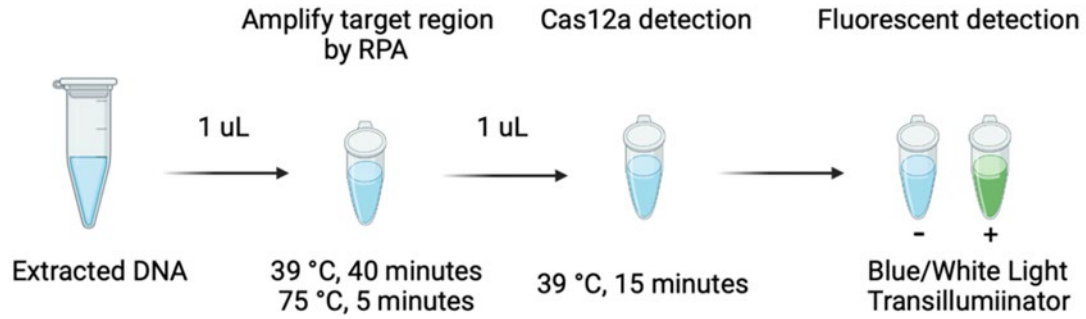

Figure 1 The workflow of the overall process for rapid detection based on RPA and CRISPR-Cas12a assay. This image is created by BioRender.com

RPA amplification of MTB rpoB gene

To test the performance of RPA primers shown in Table 1, four forward primers and two reverse primers in conserved region of the MTB rpoB gene were designed. The gel electrophoresis in Figure 2 showed that all tested primer pairs can amplify rpoB gene with high specificity. However, the primer pair of rpoB4-F x rpoB2-R was chosen for further experiments because it can also amplify other NTM species (data not shown), which could be useful for classification by CRISPR-Cas detection system.

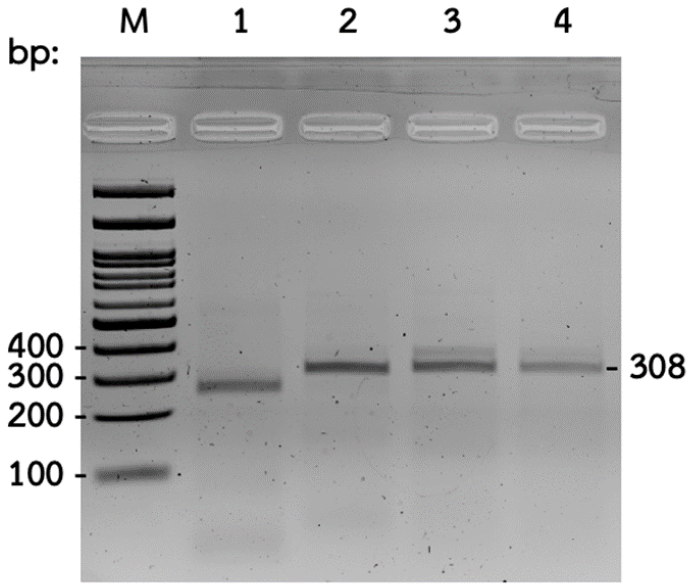

Figure 2 Gel electrophoresis of RPA products by amplification of extracted TB genomic DNA with the following primer pair: 1) rpoB1-F x rpoB1-R primer pair, 2) rpoB2-F x rpoB2-R primer pair, 3) rpoB3-F x rpoB2-R primer pair, 4) rpoB4-F x rpoB2-R primer pair. M (= marker): DNA ladder in the range from 100-3,000 bp.

CRISPR-Cas12a detection of MTB rpoB gene

To test the performance of CRISPR-Cas12a detection, TB-1 crRNA at MTB rpoB gene was designed to contain a 24 bp spacer sequence binding to the target DNA after PAM sequence 5'-TTTC-3' (Figure 3). When testing TB-1 crRNA with RPA amplified products from M. tuberculosis, the reporter fluoresced as expected. However, when using extracted DNA from M. abscessus, M. avium, M. fortuitum, M. kansasii, M. gordonae, and M. intracellulare, RPA from M. fortuitum also gave positive result with TB-1 crRNA (Figure 4a). Ooi et al. (2021) have shown that Cas12a can tolerate mismatches at position 11^{th}. By comparing rpoB sequence of M. tuberculosis and M. fortuitum, there was one mismatch at position 12^{th} of the spacer. Hence, a mismatch at the 11^{th} position of spacer (Adenine to cytosine) was introduced into the TB-1 crRNA to include more mismatches with M. fortuitum, which could potentially reduce fluorescence outcome, while still maintain fluorescence for M. tuberculosis

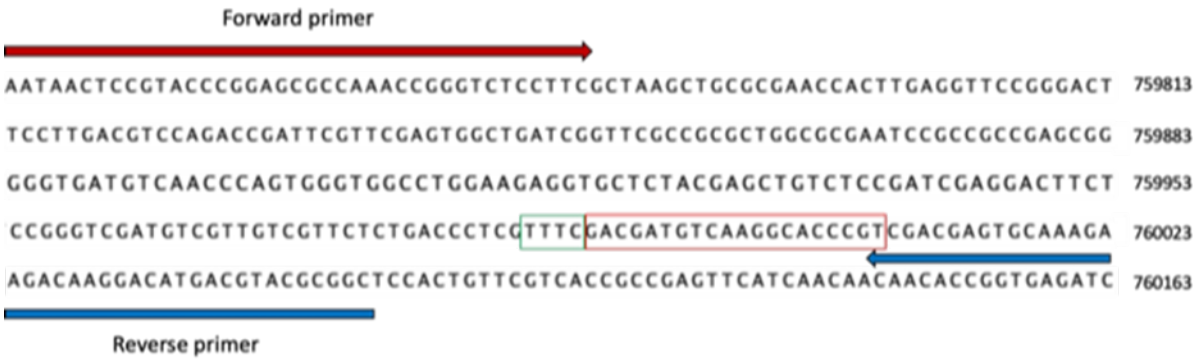

Figure 3 Design of primer and specific crRNA probe based on MTB rpoB sequence were downloaded from M. tuberculosis H37Rv. Red box represents target regions for crRNA. Green box represents the PAM motif of Cas12a. The rpoB4-F x rpoB2-R primer pair used for amplification of rpoB fragments are indicated by red and blue arrows, respectively.

After performing the cross-reactivity test with TB-2 crRNA (Figure 4a), the result showed that only extracted DNA from M. tuberculosis but not other mycobacteria yielded fluorescent signal, suggesting that TB-2 crRNA can discriminate between MTB and other NTM species. To evaluate the limit of detection (LOD), standard DNA was diluted at a concentration ranging from 10 to 10^5 copies/μL and used as a template for RPA reaction with CRISPR-Cas12a. The results revealed that the limit of detection for MTB was approximately 10^3 copies/μL as shown in Figure 4b.

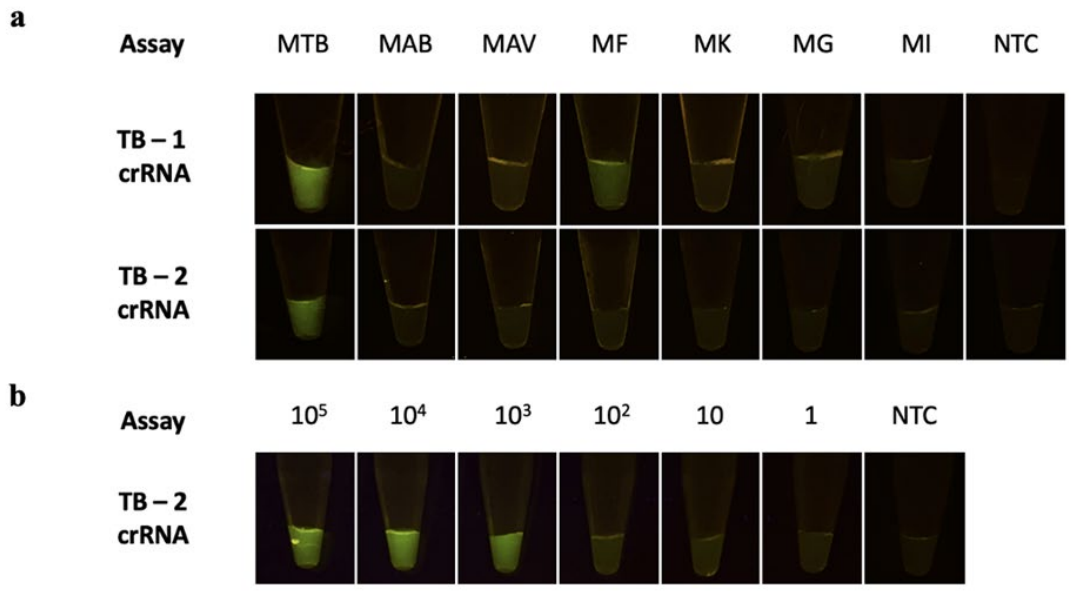

Figure 4 Detection of MTB based on CRISPR-Cas12a. (a) Cross-reactivity testing of RPA and CRISPR-Cas12a assay with TB-1 and TB-2 crRNA against other NTM species. (b) Limit of detection for MTB based on RPA and CRISPR-Cas12a assay. NTC: no template control. MTB: M. tuberculosis; MAB: M. abscessus; MAV: M. avium; MF: M. fortuitum; MK: M. kansasii; MG: M. gordonae; MI: M. intracellulare.

DISCUSSION & CONCLUSION

Tuberculosis (TB) is a global health concern that leading cause of death among infectious diseases. Therefore, early detection and identification of the M. tuberculosis (MTB) causing the disease would have a significant clinical impact since the treatment of the infection caused by MTB complex is different from that of non-tuberculosis species for helping diagnosis, would be an important factor, which could lead to early treatment and stop the disease (Gopalaswamy et al., 2020). The gold standard MTB detection and identification method is a combination of culture-based methods and molecular techniques, such as mycobacterial culture and the Xpert MTB/RIF assay, respectively (Campelo et al., 2021; Diriba et al., 2017). However, this assay is required specialized facilities and instrument that is not portable and only available in a high capital investment (Shapiro et al., 2021). Here, the present assay was developed based on RPA combined with the CRISPR-Cas12a system for portable detection of MTB (Figure 1). this technique is a portable method for detecting MTB that requires a few basic instruments comprising a heat box for RPA isothermal amplification and a BluPAD Dual LED Blue/White Light Transilluminator for the fluorescent signal observed by the naked eye and provided the turn-around time within 1 hour, which was obviously shorter than the standard method.

This study demonstrated a Limit of Detection (LOD) of MTB at 103 copies/μL. In contrast, Kim et al. (2021) reported a LOD of MTB at 104 copies/μL using their assay (Kim et al., 2021). Consequently, the combination of RPA for amplification and CRISPR-Cas12a for detection in this assay outperformed the sole use of loop-mediated isothermal amplification (LAMP). The improved LOD achieved by incorporating the CRISPR-Cas12a detection step highlights its efficacy in identifying target DNA even at low genetic material concentrations. These findings have significant implications and offer a promising alternative for the diagnosis and early detection of TB.

In addition, we successfully amplified the rpoB target gene by designing primers that conserve the region of MTB. Furthermore, these primers can be utilized to amplify the rpoB target gene of other Mycobacterium species as well. We specifically designed primers for RPA amplification that are of appropriate length, approximately 35 to 40 nucleotides, and specific to the target gene. This design enables efficient and accurate amplification of the target gene in various Mycobacterium species. This capability has significant implications in the field of molecular diagnostics and research, as it allows for the detection and identification of different Mycobacterium species using a common target gene. The conservation of the amplified region ensures the specificity and reliability of the assay (Huh et al., 2019). The designed primers provide a valuable tool for studying the rpoB gene across various Mycobacterium species. Moreover, these primer pairs can also aid in studying the specificity of detection using MTB-specific crRNA. By combining the RPA products from MTB and other Mycobacterium species rpoB target gene with crRNA specific to MTB, we can investigate the specificity of the CRISPR-based detection method.

During the specificity testing of CRISPR-Cas12a for MTB detection, it was observed that the first designed crRNA exhibited specificity towards MTB but showed cross-reactivity with M. fortuitum (Figure 4a). To improve the specificity of the MTB crRNA, we implemented a strategy based on the work of Ooi et al. (2021) (Ooi et al., 2021). TB-2 crRNA becomes more specific to the target, minimizing the occurrence of cross-reactivity with closely related species such as MF (Figure 4a). Therefore, the development of the TB-2 crRNA demonstrates the potential for tailored crRNA design in enhancing the accuracy and reliability of MTB detection using CRISPR-Cas12a technology.

Nevertheless, this study also has several limitations. One significant limitation is the absence of direct clinical specimen evaluation for the RPA combined with the CRISPR-Cas12a assay in the detection and identification of MTB. The use of clinical samples, including sputum,

blood, tissues, and bronchoalveolar lavage fluid, is crucial for assessing the performance and reliability of the assay in real-world scenarios (Kalawat et al., 2010). Furthermore, the LOD achieved in this study was relatively higher when compared to other research studies that have utilized CRISPR-Cas MTB detection methods, as reported by Sam et al. (2021) and Xu et al. (2020) (Sam et al., 2021; Xu et al., 2020). This suggests that further improvements are necessary to enhance the sensitivity and lower the LOD of the assay. To address these limitations, future investigations should prioritize the evaluation of the RPA combined with the CRISPR-Cas12a assay using clinical samples. This will provide more comprehensive and clinically relevant data on the assay's performance and feasibility in diagnosing and monitoring MTB infections. Additionally, optimization strategies should be explored to enhance the assay's sensitivity and lower the LOD, potentially through modifications in the primer design, amplification conditions, or detection protocols.

In conclusion, the development of RPA combined with CRISPR-Cas12a technology offers several advantages for MTB detection. The assay is portable, user-friendly, and provides results within a short timeframe, typically within one hour. These features make it a promising tool for point-of-care testing and rapid diagnosis of MTB infections.

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